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Culture of human embryos for studies on the derivation of human pluripotent cells: a preliminary investigation

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Abstract. Several different culture conditions were evaluated for culturing grade 4 embryos (containing 2–4 blastomeres and with >50% fragmentation) 68 h after fertilization to the blastocyst stage. Embryos were co-cultured with buffalo rat liver (BRL) cells in Menezo's B2 medium with or without 10% v/v synthetic serum substitute (SSS), co-cultured with BRL cells in KSOM with or without 10% SSS, or cultured in KSOM with 100 nM heparin binding epidermal growth factor. The most consistent development was obtained when embryos were co-cultured with BRL cells in KSOM. Rates of development to the blastocyst stage were between 27% and 40%. After reaching the blastocyst stage, continued culture of these blastocysts was only possible in a medium without serum. In a serum-deprived medium cells attached and showed initial outgrowth, but did not survive passaging. Using another approach, inner cell masses (ICMs), isolated from blastocysts with high efficiency using immunosurgery, were able to attach to a feeder layer in the presence of serum. Some ICMs differentiated whereas others could be succesfully passaged up to four times. The embryonic cells were morphologically different from murine embryonic stem cells. Instead of well-defined colonies, the human colonies were characterized by individual cells and colonies without defined borders.

Introduction

Under the appropriate conditions, isolated murine inner cell mass (ICM) cells can become transformed into embryonic stem (ES) cells, which proliferate indefinitely in an undifferentiated state in culture. In the mouse, where ES cell differentiation has been studied extensively, ES cells are derived by culturing pre-implantation embryos at the morula or blastocyst stage in enriched medium and isolating colonies of proliferating cells that maintain their capacity for differentiation (Evans and Kaufman 1981; Martin 1981). When ES cells are injected into the blastocyst cavity they can contribute to all lineages in the embryo, even the germ line (Bradley *et al.* 1984).

Because of their pluripotency, mouse ES cells have provided valuable *in vitro* models for studying differentiation of the cell lineages that form during early embryonic development. Such studies have revealed mechanisms that regulate differentiation and proliferation of these early lineages. In other studies the pluripotency of mouse ES cells has led to profound contributions in developmental genetics by providing a means of generating and selecting for mutations in specific genes (gene targeting) in mice (reviewed by Capecchi 1989; Joyner 1993; Hogan *et al.* 1994; Copp 1995). Although derivation of ES cells from several other species has been reported, including cow, hamster, pig, sheep and rabbit, to date no germline transmission from chimeric tissues derived from ES cells has been documented in those species.

Mouse ES cells have the capacity to form a wide variety of intermediate stem cells and terminally differentiated cells *in vitro*. Among the cell types recognized in ES cell cultures or embryoid bodies are derivatives of haematopoietic stem cells (nucleated erythrocytes, lymphocytes, macrophages), contracting muscle cells, neurites, and endothelial cells (reviewed by Pedersen 1994; Dushnik-Levinson and Benvenisty 1995; Keller 1995; Weiss and Orkin 1996).

The derivation and analysis of pluripotent human embryonic cells would increase our knowledge of the causes of congenital disease through an understanding of the mechanisms of early human embryonic differentiation. Because of their potential to give rise to human beings, normal human embryos are not considered appropriate subjects for certain invasive experimental studies in mammalian developmental biology (Human Embryo Research Panel 1994; National Bioethics Advisory Committee 1997). Moreover, owing to their inaccessibility and the small amount of tissue available from early human embryos, many molecular and cellular approaches are not feasible with them. Therefore, *in vitro* studies of the differentiation of pluripotent human embryonic cells would provide insight into the unique features of early human cell lineages and their differentiation mechanisms through cell and molecular studies that would otherwise not be feasible. The knowledge gained in such studies could also have a far-reaching impact on understanding the differentiation and maintenance of stem cells of later tissues and organs,

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with potential clinical applications in transplantation therapies.

A study by Bongso *et al.* (1994) is the only other documented attempt at culturing embryonal cells from human blastocysts. Embryos were allowed to attach to a feeder layer before the ICM was isolated, disaggregated and cultured for 10–14 days. The resultant colonies had a morphology similar to murine ES cells, and it was possible to obtain two passages before the cells died or differentiated into fibroblasts. Although the cells also stained positive for alkaline phosphatase (AP), no other ES cell characteristics were studied.

Success in obtaining ES cell lines from non-human primates has been reported by Thomson *et al.* (1995, 1996). Embryonic cells derived from rhesus and marmoset had a morphology more like human embryonal carcinoma (EC) cells than murine ES cells. These primate cell lines formed flat colonies with distinct individual cells, as opposed to ES cells which have piled up colonies with distinct borders (Thomson and Marshall 1998). An additional difference between mouse ES and rhesus ES cells involves the requirement for leukemia inhibitory factor (LIF). Mouse ES cells require LIF to stay in an undifferentiated state. Rhesus embryo derived cells, in contrast, are not dependent on LIF, but instead rely on another, as yet unidentified, factor produced by fetal fibroblast cells (Thomson and Marshall 1998). Rhesus ES cells maintain an undifferentiated morphology when cultured on LIF -/- feeder layers. However, they rapidly differentiate when taken off the fetal fibroblasts, even in the presence of LIF. A further distinction between murine and primate ES cells is their repertoire of markers for pluripotency; AP is the only marker for pluripotent cells that both mouse and rhesus cells have in common. All other tested markers for pluripotency were unique to murine cells (e.g. SSEA-1) or to primate cells (e.g. SSEA-3, SSEA-4. TRA-1-60. TRA-1-80) (Thomson and Marshall 1998). As is the case for murine cells, rhesus ES cells differentiate into tissues from all three lineages, both *in vitro* and after injection into immunodeficient mice. Especially, the differentiation of rhesus ES cells in immunodeficient mice is extensive and remarkable, with complex structures like toothbuds developing (Thomson and Marshall 1998). Thus, although the rhesus cells show major differences compared with mouse ES cells in morphology, growth requirements and markers, their *in vitro* and *in vivo* differentiation characteristics conclusively show they are pluripotent.

A major impediment to the development of embryo-derived pluripotent human cell lines is the restricted access to human embryos for research. The main access to human embryos for experimental purposes are embryos that are rejected for clinical therapy of infertility. Here we describe the establishment of culture conditions to obtain blastocysts and isolate ICM for research from these donated embryos and present our initial studies in deriving embryonic cell lines.

Materials and methods

Embryo donation

All the research embryos were donated by patients undergoing treatment in the *in vitro* fertilization (IVF) program at the University of California, San Francisco, according to protocols approved by the institutional Committee on Human Research. The donated embryos fall into one of two categories: chromosomally abnormal (1 or 3 pronuclei (PN)) or poor quality, chromosomally normal (2 PN) embryos. The number of PN are counted 16–20 h after fertilization. The decision on which embryos will be transferred to the patient or cryopreserved is generally made 68 h after fertilization. To facilitate this process the embryos are graded according to a 5-point grading system, with grade 1 being the best embryos (Dawson *et al.* 1987). Grade 1 embryos have even-sized blastomeres with no fragmentation, grade 2 embryos have less than 10% fragmentation or uneven blastomeres, grade 3 embryos have between 10 and 50% fragmentation, and grade 4 embryos have >50% fragmentation. Experience has shown that grade 4 embryos rarely establish a pregnancy when transferred back to the patient, nor do they survive cryopreservation. Grade 5 embryos are totally fragmented or degenerated. The embryos donated for the present research were either grade 4 with 2, 3 or 4 blastomeres or grade 5.

Embryo culture

Prior to release into the present study, the oocytes were inseminated and cultured for 16–20 h in 1 mL Earle's medium (Conaghan *et al.* 1993) with 10% v/v synthetic serum substitute (SSS; Irvine Scientific, Santa Ana, CA, USA). Zygotes were transferred into 50 μL of the same medium or KSOM (Speciality Media, Lavalette, NY, USA) under mineral oil (Sigma, St Louis, MO, USA) and cultured for a further 48 h. After release into the study, embryos from each patient were pooled and cultured together in 50 μL droplets of medium under oil. All cultures were in a gas phase of 5% CO_2 in air at 37°C. The following different culture conditions were used: (*1*) co-culture with buffalo rat liver cells (BRL: ATCC, Rockville, MD, USA) in Menezo's B2 medium (Fertility Technologies, Inc., San Diego, CA, USA) with or without 10% v/v SSS; (*2*) co-culture with BRL cells in KSOM with or without 10% v/v SSS; or (*3*) culture in KSOM with 100 nM heparin binding epidermal growth factor (hbEGF: R&D Systems, Minneapolis, MN, USA).

Preparation of BRL cells

Fifty microliter droplets of M199 (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% v/v fetal calf serum (FCS), 100 I.U. penicillin and 100 μg streptomycin (all from Sigma) were layered under oil. Cryopreserved BRL cells were thawed in M199 with 25 mM HEPES (Life Technologies) and seeded directly into the culture droplets. When the BRL cells reached confluence within the drop, the medium was replaced with one of the embryo culture media described earlier. All media were equilibrated at least 2 h before the introduction of embryos to the droplets.

Blastocyst culture

The zona pellucida was removed from the blastocysts by incubation in 0.5% pronase (Sigma) in phosphate-buffered saline (PBS: Life Technologies), and whole embryos were either placed in standard ES cell medium (Robertson 1987), which contains 10% FCS (HyClone, Logan, UT, USA) and 10% newborn calf serum (Sigma) or they were placed in serum-deprived medium (SDM) consisting of Dulbecco's modified Eagles's medium supplemented with 2% BSA, 10 μg mL^{-1} insulin (both from Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), 200 μg mL^{-1} transferrin, 20 μg mL^{-1} LDL, Pen/Strep, 1 × non-essential amino acids and 7.5×10^{-5} M monothioglycerol (all from Sigma).

ICM isolation

ICMs were isolated by immunosurgery, according to the method of Solter and Knowles (1975). Embryos were incubated for 20–30 min in a rabbit anti-human antibody (generously donated by James Thomson, University of Wisconsin, Madison, WI, USA) then washed and incubated in guinea pig complement (Sigma) for 30 min to induce lysis. The trophectoderm was removed by pipetting the embryos through a small-bore pipette. The isolated ICM was then plated onto a feeder layer of either mitomycin-C inactivated STO (ATCC) or fetal fibroblast cells in standard ES cell medium. Outgrowths were passaged in Ca^{2+}/Mg^{2+} free PBS supplemented with 0.5 mM EDTA (Sigma).

Results

Chromosomally abnormal embryos

Blastocyst development from 1- and 3- or 4-PN embryos after co-culture in either KSOM or B2, both supplemented with 10% SSS, was similarly poor. In B2 medium 2/15 embryos with 1 PN and 1/37 embryos with 3 or 4 PNs developed to the blastocyst stage. In KSOM medium, 2/13 and 1/25 embryos with 1 PN and 3–4 PN, respectively, developed to the blastocyst stage.

Normally fertilized grade 4 embryos

The development of grade 4 embryos was better in the simple KSOM medium than in the complex B2 medium (Table 1). Supplementation with 10% SSS did not seem to influence the development to the blastocyst stage. Although KSOM medium with 100 μM hbEGF also supported development to the blastocyst stage at reasonable rates, the development obtained with KSOM and BRL co-culture seemed to be more consistent (Table 1). In a few instances chromosomally normal and abnormal embryos of a patient were pooled and their development to the blastocyst stage could therefore not be separated (Table 1). Because our goal was to obtain as many blastocyst as possible for further experimental use, the BRL co-culture system with KSOM medium without any supplementation was selected for further embryo culture (Table 2).

Table 1. Blastocyst development of grade 4 embryos after culture in different culture conditions

Culture condition	No. embryos	No. blastocysts
B2 + BRL (+ SSS)	25	1
B2 + BRL	15	0
KSOM + BRL (+ SSS)	41	16
KSOM + BRL	62[A]	25
KSOM + hbEGF	35[B]	9

[A] Includes 12 chromosomally abnormal embryos; [B] includes 3 chromosomally abnormal embryos.
BRL, buffalo rat liver cells; SSS, synthetic serum substitute; hbEGF, heparin binding epidermal growth factor.

Immunosurgery

In the majority of the blastocysts an ICM could be successfully isolated with immunosurgery. The isolation of an ICM indicates that initial differentiation had occurred. Although cell counts were not performed, the size of the ICMs did not seem to correlate to the size of the embryos. In a few large embryos, very small ICMs were isolated, whereas in some smaller embryos, bigger ICMs were isolated.

Blastocyst culture

The first blastocysts obtained were cultured in standard, serum-containing, ES cell medium. No further development of these blastocysts was obtained. When embryos were cultured whole in SDM, the blastocysts attached and showed initial outgrowth, but eventually all differentiated or died. Passage was not successful in any of these outgrowths.

After immunosurgery, the ICMs attached and were able to be passaged (Table 2). Cultures of two ICMs were passaged three and four times respectively. Both cultures differentiated before any pluripotent characteristics could be determined. Differentiation was characterized by large multinucleated cells. The second cell line was investigated to determine its human origins, and it was shown to be a human X,Y cell line by fluorescent *in situ* hybridization (Penketh *et al.* 1989) with a human X,Y probe (Vysis, Downers Grove, IL, USA). The morphology of the cell lines did not resemble mouse ES cell lines. Instead of well-defined colonies, the human colonies were characterized by individual cells and colonies without defined borders.

Discussion

The results in the study reported here were obtained over a 1.5-year period. The inconsistency and rarity of human embryos makes designing and replicating experiments difficult or impossible and therefore embryos were not randomized between treatments. Also, although patient age is a major factor in the developmental potential of the resultant

Table 2. Blastocyst development of donated human embryos in KSOM on buffalo rat liver cells, subsequent inner cell mass (ICM) isolation and culture in standard embryonic stem cell medium

No. embryos released	146
Blastocyst after zona pellucida removal	40[A]
ICM isolated	32[B]
ICM attached	21[C]
Attached ICMs differentiated	11
1st passage	8
2nd passage	5
3rd passage	2

[A] Four embryos were lost in the process of moving the embryos between laboratories and isolation of the ICM; [B] two embryos had attached before the ICM could be isolated; [C] three cultures were contaminated and could not be evaluated for ICM attachment.

embryo (Munné *et al.* 1995; Rosenwaks *et al.* 1995), no adjustments were made for the patient's age in calculating and comparing the results.

Few chromosomally abnormal embryos, as determined by the presence of 1, 3 or 4 PNs after fertilization, reached the blastocyst stage. We started culturing chromosomally abnormal embryos because it has been reported that 1-PN embryos can be diploid (Levron *et al.* 1995; Palermo *et al.* 1995). However, on the basis of the poor development obtained when culturing these embryos, it was decided not to continue using these embryos for this research.

Grade 4 embryos, scored 3 days after fertilization, can, under optimal conditions, develop to the blastocyst stage. Most blastocysts were recovered after a total culture of 5–7 days (post insemination). There was a substantial difference in development to the blastocyst stage when KSOM or B2 was used. KSOM is a simple salt solution characterized by a low osmolarity (256 mOsm) and a low glucose concentration (0.2 mM). B2, in contrast, is a complex medium and has a high glucose concentration (5.5 mM). We have been impressed by the good development of human embryos in KSOM, considering that this medium was developed for embryo culture in the mouse. Other recent reports indicate that a two-media system improves development of human embryos to the blastocyst stage (Gardner 1998). Three days after fertilization, the embryos are placed in a medium with a higher glucose concentration, based on the observation that embryos start utilizing glucose at the 8-cell stage (Hardy *et al.* 1989). Considering the good development rate to the blastocyst stage in the present study, the embryo must use alternative substrates (e.g. pyruvate, glutamine, lactate).

Although it is common practice to obtain murine ES cells (Robertson 1987) by plating the whole blastocyst and then retrieving ES cell colonies, this approach was not successful with human embryos. There was, however, a difference when whole embryos were cultured in standard ES cell medium, which contains 20% serum, or in SDM with BSA. In serum-containing medium, embryos did not attach and subsequently degenerated. In the SDM, embryos attached and showed initial outgrowth. However, none of these outgrowths survived after passage. Culture of isolated ICMs in the presence of serum was more successful. Although more than half of the cultures differentiated, several cultures were successfully passaged.

Approximately 20–25% of human embryos show chromosomal abnormalities (Jamieson *et al.* 1994). It is also conceivable that grade 4 embryos suffer from a greater frequency of chromosomal abnormalities than grade 1 embryos (Munné *et al.* 1995). Because some ICMs did grow and extend whereas others died shortly after attachment, and although culture conditions have not been refined for human isolated ICMs, the original quality of the embryo and associated chromosomal abnormalities will, most probably, be a cause in the early demise of some ICMs.

Although KSOM with BRL co-culture allows grade 4 embryos to develop to the blastocyst stage, we have limited indication as to the quality of these blastocysts. When rejected embryos are used for research, the interpretation of the results as they pertain to clinical embryos, is not straightforward. The relevance and impact of these results on clinical embryos is unknown, but given the paucity of human embryonic material available for experimental studies, the rescue of poor grade embryos represents a valuable resource.

In summary, we have shown that embryos, containing 2–4 blastomeres and with >50% fragmentation, 68 h after fertilization, can, under the right conditions, develop into blastocysts. From these blastocysts an ICM can be isolated, indicating that these embryos are capable of initial differentiation. The resulting ICMs are acceptable starting material for studies in the derivation of pluripotent cells.

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Addendum

While this manuscript was under review J. Thompson and colleagues published their work on the derivation of human ES cell lines (*Science* 1998; **282:** 1145–7).

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